isolation by a novel method (RIN > 6 = 70%, 260/280: 1.8~2.0 = 100%, 260/230 > 1.0 = 95% (n=50)) and morphometry calculation by μCT (OA n=20, non-OA normal n=2), Illumina microarray was performed (OA n=10, non-OA normal n=2) and real-time quantitative polymerase chain reaction was executed for data validation (OA n=20, non-OA normal n=2).
Results: A total of 205 transcripts were found be expressed at significantly different level among the regions. Most of these genes related to the extracellular matrix synthesis, cell proliferation, bone cell differentiation. These genes might play key roles in breakdown of subchondral bone homeostasis in OA.
Conclusions: The novel USB RNA isolation methodical approach reported here not only allows to determine area-specific gene profiles in knee specimen. In addition, this study also show some genes might play a role in OA bone remodeling.

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CHARACTERIZATION OF BONE CELLS FROM HEALTHY AND OSTEOARTHRITIS PATIENTS

G. Lisignoli, C. Manferdini, E. Gabusi, F. Grassi, A. Piacentini, B. Grigolo, L. Cattini, A. Facchini
Istituto Ortopedico Rizzoli, Bologna, Italy

Human bone cells derived from bone marrow (BM) aspirates or trabecular bone (TB) tissue have been used for studies of bone biology. However, an in-vitro characterization of these cells is necessary not only for basic studies in health and disease but also for cellular therapies for tissue repair. Bone tissue is often discarded during joint replacement or bone correction surgery and this tissues could represent a good potential alternative source of osteoprogenitor cells for tissue engineering purposes. The study aims to validate the potential of different bone cells obtained from the same anatomical site. Therefore, we isolated MSCs from BM and osteoblasts (OBs) from tibia of healthy and OA patients. MSCs and OBs were compared for growth conditions, cell proliferation, phenotype and osteogenic potential. MSCs and OBs were grown both in α-MEM and DMEM/F12 medium. We found that MSCs were able to grow only in α-MEM while OBs could survive and proliferate in both medium tested. The proliferation of healthy MSC and OB grown in α-MEM was significantly higher than OA MSC and OB, while no significant differences were observed in OB grown in DMEM/F12. Flow cytometric analysis for CD73, CD90, CD105, CD146, alkaline phosphatase, bone sialoprotein (BSP) and collagen type I did not show differences among the groups analysed. The osteogenic potential of MSC and OB grown in α-MEM were next tested inducing the cells to differentiate along the osteoblast lineage in osteogenic medium and demonstrating the positive staining for extracellular calcium deposition at day 28. Real time PCR analysis demonstrated a decrease expression of alkaline phosphatase and collagen type I associated with an increase of collagen type XV from Day 0 to day 28, while BSP expression was significantly increased only in healthy MSC and OA OB but not in OA MSC. Osteogenic control experiments were also performed using MSC from iliac crest. Here, we found a decreased expression of alkaline phosphatase and collagen type I from day 0 to day 28 and a significant increase in collagen type XV and BSP expression. These data suggest that even if osteogenic differentiation occurs in MSC and OB from OA patients, OA OB were skewed towards a pattern of expression of specific osteogenic markers more similar to that found in canonical BM MSC.

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SMAD2/3 SIGNALING IS CRUCIAL FOR CARTILAGE MAINTENANCE, WHEREAS SMAD1/5/8 SIGNALING DETERMINES CHONDROCYTE TERMINAL DIFFERENTIATION

E.N. Blaney Davidson[1,2], C.A. Hellingman[3], W. Koevoet[3], E.L. Vitters[1], W.B. van den Berg[1], G.J. van Osch[3], P.M. van der Kraan[1]
[1]Univ. Med. Ctr. St. Radboud, Nijmegen, Netherlands; [2]Leiden Univ. Med. Ctr., Leiden, Netherlands; [3]Erasmus Med. Ctr., Rotterdam, Netherlands

Purpose: In osteoarthritis (OA), chondrocyte behavior seems a recapitulation of the developmental process. Chondrocytes loose their articular cartilage phenotype and progress into a state highly similar to chondrocyte hypertrophy, overexpressing MMP13. We have previously shown that during OA, the balance between the TGF-beta receptors ALK1 and ALK5 shifts, favoring the ALK1 receptor. Downstream this leads to a shift from signaling via Smad2/3 towards Smad1/5/8, thereby favoring the signaling pathway that is important in terminal differentiation. As a consequence, blocking of the Smad1/5/8 pathway in differentiating chondrocytes might be a tool to inhibit chondrocytes terminal differentiation.
We show that Smad1/5/8P expression is correlated with MMP13 expression in limb formation and in terminally differentiated pellet cultures. Moreover, whereas blocking Smad2/3P halted further chondrogenesis, we show that blocking the Smad1/5/8 pathway blocked terminal differentiation and therefore inhibited chondrocyte hypertrophy.
Methods: BMSC were chondrogenically differentiated in pellet culture in chondrogenic medium including TGF-β2. SB-505124 was added to inhibit Smad2/3 phosphorylation, while dorsomorphin was added to inhibit Smad1/5/8 phosphorylation.
Immunohistochemistry and gene-expression analysis was performed for markers of chondrogenic and terminal differentiation, as well as for Smad2/3P and Smad1/5/8P. The ability to mineralize the tissue-engineered constructs was studied *in-vitro* by adding 10 mM β-glycerophosphate (BGP) to the medium.
Results: In pellet cultures of terminally differentiated BMSCs as well as well as during embryonic limb formation, terminally differentiated chondrocytes were positive for both Smad2/3P and Smad1/5/8P. On the other hand, hyaline-like cartilage that lacks expression of MMP13 and collagen X did not express Smad1/5/8P, but expressed Smad2/3P.
To investigate whether both pathways were important during onset of chondrogenesis, SB-505124 or dorsomorphin were added throughout the culture period. This resulted in lack of collagen II expression, suggesting that both pathways are involved in early chondrogenesis. However, when the chondrocytes were given the opportunity to first differentiate towards a cartilage phenotype for 14 days and thereafter either Smad2/3 or Smad1/5/8 phosphorylation were blocked until day 35 distinct functions were demonstrated.
Blocking Smad2/3phosphorylation from day 14-35 resulted in a halt in collagen II production, whereas blocking Smad1/5/8phosphorylation decreased the expression of MMP13, collagen X and alkaline phosphatase without inhibiting further collagen II production. Blocking Smad1/5/8phosphorylationcompletely prevented mineralization (Figure 1).

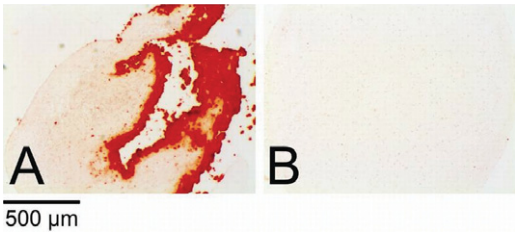

Figure 1. Alizarine Red staining demonstrating mineralization in control pellets (A), and no mineralization in pellets treated with dorsomorphin from day 14 on (B).

Conclusions: Our data show that both the Smad2/3 and Smad1/5/8 pathway are important for initial chondrogenesis in vitro. Blocking Smad2/3 after onset of chondrogenesis halts further collagen type II production suggesting this pathway is crucial for cartilage maintenance. In contrast, blocking Smad1/5/8 phosphorylation totally prevents terminal differentiation as the expression of MMP13, collagen X and Alkaline phosphatase are inhibited.

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WNT-6 INDUCES THE CHONDROGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

C. Bony, D. Guerit, C. Jorgensen Sr., **D. Noel Sr.**
Inserm, Montpellier, France

Purpose: Mesenchymal stem cells (MSC) are suitable sources for cell-based therapies in tissue engineering thanks to their capacities of differentiation towards several lineages of the skeletal tissue. In particular, their potential to differentiate into chondrocytes makes them attractive for cartilage repair. However, regeneration of a fully functional and mature tissue will rely on the use of an optimal combination of scaffold and differentiation factor. Identification of a factor that may induce the chondrocytic lineage and

maintain the mature chondrocyte phenotype avoiding terminal differentiation towards hypertrophy represents a major issue and is the objective of this study.

Methods: Bone marrow-derived murine MSCs were induced to differentiate towards chondrocytes using the micropellet culture technique in presence of Wnt-6 containing conditioned medium (CM). CM was obtained after incubation of a chondrogenic medium consisting of DMEM supplemented with ITS, proline, ascorbic acid and sodium pyruvate for 48h on confluent NIH-3T3 cells stably transfected to secrete Wnt-6. As controls, CM from NIH-3T3 cells or BMP-2 containing medium was used. Similar conditions were used to obtain CM with osteogenic or adipogenic media. After 21 days, quantitative RT-PCR was performed on total RNA to detect the expression of markers specific for each lineage and staining specific for proteoglycans, mineralization or lipid droplets was performed. Western blotting with anti-β-catenin, anti-JNK or anti-PKC antibodies was performed after migration and transfer on nitrocellulose membranes of 20 μg total proteins extracted from pellets after a time course exposure to the different CM.

Results: Pellet culture of murine MSCs in presence of NIH-derived CM or chondrogenic medium alone did not up-regulate the chondrocytic markers. On the contrary, Wnt-6 containing CM was sufficient to induce the differentiation of MSCs into chondrocytes as shown by the induction of collagen type IIB, aggrecan and COMP and a positive staining for proteoglycans. The expression levels of the transcripts were lower than those induced by BMP-2. However, contrary to BMP-2, we observed the lack of induction of the hypertrophic markers collagen type X and alkaline phosphatase when MSCs were cultured in Wnt-6 CM. Interestingly, in osteogenic or adipogenic conditions, MSCs did not up-regulate the markers specific for osteoblasts or adipocytes and rather decreased their expression level. The up-regulation of chondrocytic markers by Wnt-6 was associated with a lack of induction of the β-catenin or JNK pathways and preliminary results suggest that PKC signalling may be induced.

Conclusions: Our results suggest that Wnt-6 is one new chondrogenic factor sufficient to specifically induce the generation of chondrocytes and inhibiting their terminal differentiation. Preliminary results suggest that Wnt-6 might induce the PKC-dependent pathway to activate the chondrocyte-specific genes.

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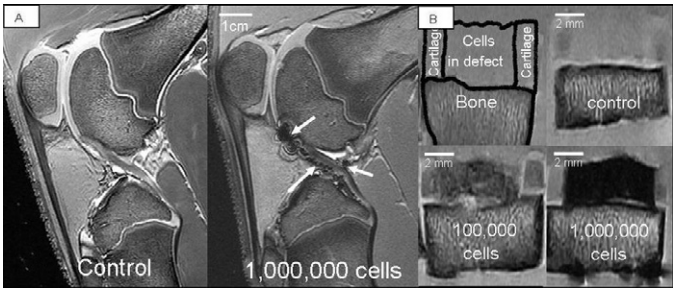
CELL TRACKING FOR CARTILAGE REPAIR USING SUPERPARAMAGNETIC IRON OXIDES: CLINICAL POTENTIAL

G.M. van Buul, G. Kotek, P.A. Wielopolski, E. Farrell, P.K. Bos, H. Weinans, J.A. Verhaar, G.P. Krestin, M.R. Bernsen, **G.J. van Osch**
Erasmus MC, Rotterdam, Netherlands

Purpose: Human bone marrow stromal cells (hBMSCs) are experimentally being used in patients as a cell-based therapy for cartilage repair. To verify the safety and efficacy of such approaches it is necessary to determine the fate of these implanted cells. Cell labeling using superparamagnetic iron oxides (SPIOs) enables non-invasive in vivo cell tracking by MRI, and has already been used in a clinical setting in various fields. In this study we describe a major step towards application of SPIO-labeling for cell tracking in clinical cell-based cartilage repair approaches. We investigated the safety, intra-articular MRI traceability and the possibility of SPIO re-uptake of this cell tracking technique.

Methods: *Safety:* hBMSCs from three donors were labeled in triplicate samples with ferumoxides (Endorem®)-protamine sulphate complexes at doses ranging from 0 - 250 μg iron/ml. After incubation for 24 hours, cell viability was assessed using a trypan blue exclusion assay. Subsequently, metabolic cell activity was quantified using the AlamarBlue® assay up to seven days after labeling. Chondrogenic capacity of hBMSCs labeled with 100 μg/ml SPIO was evaluated using thionine staining and collagen type II immunohistochemistry.
Intra-articular imaging: SPIO-labeled hBMSCs (100,000 to 5,000,000 cells) were injected ex vivo in pig knees, to mimic a clinically relevant sized model. Furthermore, SPIO-labeled cells (10,000 - 1,000,000 per 75 μl) were seeded in cartilage defects in vitro. Scanning was performed on a clinical 3.0 T MRI scanner.
SPIO re-uptake: To study possible SPIO re-uptake by synovial cells, viable and dead GFP-SPIO double-labeled chondrocytes were seeded on human synovium explants. After co-culturing for five days, samples were harvested and analyzed using fluorescence- and light microscopy.

Results: *Safety:* SPIO labeling resulted in labeling efficiencies of ± 95% and did not impair cell viability or subsequent cell activity at any dose. SPIO-labeled hBMSCs produced amounts of glycosaminoglycan and collagen type II comparable to unlabeled control cells.
Intra-articular imaging: All SPIO-labeled cell dosages, both intra-articularly injected and cells seeded in cartilage defects, were visualized by MRI (Fig. 1). Cell-dose dependent signal voids were observed, and cells could be clearly differentiated from anatomical structures. SPIO-labeled cells seeded in cartilage defects could be quantified using a T2* mapping MRI technique.
SPIO re-uptake: GFP^+-$SPIO^+$ cells, indicating originally seeded cells, were seen in samples containing live cells. GFP^--$SPIO^+$ cells, indicating SPIO re-uptake by synovial cells, were found in samples containing dead cells.



Conclusions: hBMSC labeling with SPIO particles is feasible, without leading to negative effects on cell viability, subsequent metabolic cell activity or chondrogenic differentiation. SPIO-labeled cells can be visualized intra-articularly by MRI and quantified when seeded in a cartilage defect. Although possible SPIO re-uptake by host cells has to be taken into account, we showed promising results for the use of SPIO labeling for cell tracking in clinical cartilage repair. This approach provides the extra advantage to simultaneously track cells and evaluate cartilage repair in one MRI session.

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CHONDROGENIC POTENTIAL OF SUBPOPULATIONS OF CELLS EXPRESSING MESENCHYMAL STEM CELL MARKERS DERIVED FROM HUMAN SYNOVIAL MEMBRANES

M.C. Arufe[1,2], A. De la Fuente[1], S. Díaz[1,2], I. Fuentes[1,2], F.J. De Toro[1,2], F.J. Blanco[1,3]
[1]Osteoarticular and Aging Res. Lab. Cellular Therapy Unit. Ciber-BBN. INIBIC-Complejo Hosp. Univ. A Coruña, A Coruña, Spain; [2]Dept. Med. Area of Anatomy and Human Embryology. Fac. Hlth.Sci. Univ. of A Coruña, A Coruña, Spain; [3]Cathedra BIOIBERICA of Cell Therapy Univ. of A Coruña, A Coruña, Spain

Purpose: Synovial membrane mesenchymal stem cells (MSCs) have been demonstrated to be a good source of cells for the study of cartilage tissue engineering. Multiple stem cells markers have been found by flow cytometry and immunofluorescence in MSCs from human synovial membrane pools. In this study we analyzed the chondrogenic potential of subpopulations of MSCs derived from human synovial membranes enriched for CD73, CD106 and CD271 markers.

Methods: Subpopulations of human synovial membrane MSCs enriched for CD73, CD106 and CD271 markers were isolated using a cytometry sorter and characterized by flow cytometry for MSC markers. The expression of Sox9, Nanog and Runx2 genes by these cells was measured by reverse transcriptase-polymerase chain reaction. The chondrogenesis of each subpopulation was assessed by culturing the cells in a defined medium to produce spontaneous spheroid formation and differentiation towards chondrocyte-like cells. The examination of the spheroids by histological and immunohistochemical analyses for collagen type II (COL2), aggrecan, collagen type I (COL1), metalloprotease 13 (MMP13) and collagen type X (COLX) levels were performed to assess their chondrogenesis capacity. The adipogenesis and osteogenesis potential of each subpopulation was determined using commercial media; the resulting cells were stained with oil red O or red alizarin to test the degree of differentiation.

Results: The subpopulations had different profiles of cells positive for the MSC markers CD44, CD69, CD73, CD90 and CD105 and showed different expression levels of the genes Sox9, Nanog, Runx2 involved in chondrogenesis, undifferentiation and osteoblastogenesis, respectively. Immunohistochemical analysis demonstrated that COL1, COL2, COLX, MMP13 and aggrecan were expressed in the spheroids as soon as 14 days of culture. The $CD271^+$ subpopulation expressed the highest levels of COL2 staining